Environmental Health Perspectives
Vol. 37, pp. 117-123, 1981

Bioassaying for Ozone With Pollen Systems

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Sensitivity to ozone of pollen germinating *in vitro* is closely correlated with ozone sensitivity of the pollen parent. Ozone-sensitive and tolerant pollen populations have been identified in tobacco, petunia, and tomato cultivars. The rate of tube elongation can be reversibly slowed or stopped by exposure to low concentrations of ozone. Tube growth rates in the presence of a range of ozone dosages, of pollen populations exhibiting differing ozone sensitivity can be measured and different growth rates can be correlated with ozone dosages. The performance of selected pollen populations can then be used to bioassay ozone in ambient air by introducing the air sample into a growth chamber where ozone-sensitive pollen in growing. Petunia and tobacco pollen are especially useful because they store well at ordinary freezer temperatures and do not require special preparation prior to storage. Modified Brewbacker's growth medium is suitable for growth of both these pollen types. Four useful cultivars are Bel W-3, ozone-sensitive and Bel B, ozone-tolerant tobacco, and White Bountiful, ozone-sensitive and Blue Lagoon, ozone-tolerant petunia. Observations can be made directly by using a TV scanner, or by time lapse or interval photography. Year-round pollen production can be achieved in the greenhouse. Harvested pollen can be tested, packaged, and transported to user facilities without loss of vigor. Pollen populations are inexpensive to produce, respond reliably, and are simple to use as a bioassay for air quality.

In a previous paper (*1*) this author alluded to the fact that pollen populations differed in ozone sensitivity and that these differences were closely related to the ozone sensitivity of the pollen parent (source) (*2, 3*). SO_2 (*4, 5*) and fluorides (*6-9*) also cause a reduction in germination and tube elongation indicating that pollen behavior *in vitro* can be modified or influenced by several gases generally considered to be environmental pollutants.

Species and cultivar differences in ozone sensitivity have been well documented and are now often considered when evaluating the horticultural worth of plant materials (*10*). Differences in cultivar tolerance to ozone injury have been noted in alfalfa (*11*), eastern white pine (*12*), other coniferous species (*13*), beans (*14, 15*), sweet corn (*16-18*), cucumber (*19*), eggplant (*20*), grape (*21*), onion (*22*), pea (*23*), petunia (*24*), safflower (*25*), soybean (*26*), spinach (*27*), tobacco (*28-30*), tomato (*31-33*), turf grasses, especially *Poa Pratensis* L. (*34*), woody ornamentals (*35*), and forest tree species (*36*). This is only a partial list of those plant species showing differences in cultivar sensitivity to ozone, but it is broad enough to demonstrate that the condition is widespread. It also suggests the possibility of using this diversity as expressed through the pollen populations, to devise a system for measuring air quality by observing pollen performance *in vitro*. This paper attempts to illustrate how that might to done for the gaseous photochemical pollutant ozone(O_3).

Background and General Procedures

Among the many plants that can be chosen as pollen sources, it was important to us species and cultivars which were known to be sensitive to ozone at levels encountered in ambient air and whose pollen would respond *in vitro* to those same ozone concentrations. Tobacco, petunia, and tomato were among the logical choices, since pollen sensitivity to ozone had been demonstrated in several cultivars of these species (*2, 3, 5, 37*). *Nicotiana tabacum* cv

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Bel W-3, *Petunia hybrida* cv White Cascade (or White Bountiful), and *Lycopersicum esculentum* cv Tiny Tim are all very sensitive to ozone injury and produce pollen populations which show the same degree of sensitivity. *N. tabacum* cv Bel B, *Petunia hybrida* cv Blue Lagoon, and *Lycopersicum esculentum* cv Heinz 1439 all tolerate higher levels of ozone and produce ozone-tolerant pollen populations (*2, 3, 31-33, 37, 38*). There are other cultivars of tobacco, petunia, and tomato which might serve as well as pollen sources, but their pollen/ozone responses have not yet been studied. Also, clones of eastern white pine, *Pinus strobus* L. would be potential pollen sources because of known differences in ozone sensitivity among individual trees and because they produce huge amounts of pollen which is easily stored and retains its viability over many years. However, to date, no studies have been made on the *in vitro* pollen/ozone response. Sweet corn also shows great diversity in ozone response but the pollen cannot be stored thus rendering the material useless in the present scheme.

The choice for this study were the cultivars of tobacco and petunia because the pollen/ozone response was understood, the plants are easy to raise from seed in the greenhouse, and they flower profusely. Large amounts of pollen are easily harvested and stored at –10°C. Germination percentage is high (80-95%) and tube elongation is fairly rapid and easily measured (*2, 3, 37, 39*).

Seed populations of tobacco cv Bel W-3 and cv Bel B, and petunia cv White Cascade and cv Blue Lagoon were sown in Vermiculite and germinated under continous light at 25°C in a green house receiving only ozone-free charcoal filtered air. Seedlings were watered with half strength Hoagland's solution, transplanted to individual "Jiffy Pots" at the appropriate time and watered with half strength Hoagland's solution until they were ready to be transplanted into regular pots. Seedings were made at regular intervals to insure that new plants were always available for harvest and old plants could be routinely destroyed to minimize the incidence of virus infection and reduce the white fly population without having to resort to spraying which might effect pollen production and viability. Seedlings were transplanted into pots containing a standard peat, sand, soil 1:1:1 mix and were grown to maturity is special designed greenhouses which received only charcoal-filtered, ozone-free air. Plants were fertilized weekly with 15:15:15 commercial liquid fertilizer. Pollen was harvested between 1100 and 1300 hours each day by removing the entire flower, carrying it into the laboratory and dissecting the pollen out of the dehiscing anthers with a steel needle into a 35 mm plastic petri dish. The dish was taped closed, coded and placed in a commercial refrigerator freezer at –10°C. Stored pollen was distributed among several dishes so that the same pollen pile was not repeatedly exposed to the ambient air when pollen was removed for study or experimentation. (For large scale application of this system, pollen could be stored in individual, sealed plastic vials). Pollen was removed from the dishes with a clean camel's hair brush as needed and dusted on to the surface of a modified Brewbacker-Quack growth medium (*40*) fortified with 0.75% Difco-Bacto Agar.

To establish the sensitivity of the pollen/ozone response, pollen was deposited on agar discs or on agar dispensed in 35 mm petri dishes. These were placed on a styrofoam disc floated on water and rotating in a small exposure chamber (*2, 3, 37, 40*). Ozone generated from a GE quartz ozone bulb (GE S411) was allowed to flow through the chamber at a rate of 1 min. The ozone concentration in the chamber was controlled by regulating the voltage passing through the quartz bulb and was continuously monitored using a Mast ozone meter with the probe centered over the rotating agar surface. Pollen germination and tube elongation were arrested at the desired point by removing the discs from the chamber and immediately placing them in a refrigerator. Discs or random sectors of the agar dish surfaces were then photographed through a microscope at 100 × and the pictures enlarged to allow examination and measurement of germination rate and tube lengths.

Studies using this system established the nature of the pollen/ozone response for tobacco, petunia, and tomato cultivars and also showed that pollen becomes ozone-sensitive only after the emergence of the tube, at least at the low ozone concentrations studied (*37*). By using a micro-observation/exposure chamber and time-lapse cinematography (unpublished 16 mm film sequence), it was demonstrated that a 10-30 sec exposure of a pollen tube of tobacco, Bel W-3 to 0.25 ppm ozone caused a reversible cessation of tube growth without interfering with tube cyclosis, again demonstrating the ozone-sensitivity of the growing tube. In a further modification, pollen behavior in the microchamber was observed by mounting a TV video camera over the microscope and germination, tube elongation, exposure sequence, and tube modification was monitored directly on the TV screen. This system has the advantage of eliminating the high cost of film processing and the uncertainty as to whether the desired sequences have been captured on film. However, without a video tape, there is no permanent record of the events for later use and interpretation.

Procedure for Pollen Bioassay

Pollen is produced, harvested and stored as previously described. Air samples are collected in 50-100 ml Teflon or pyrex glass syringes fitted with micro valves to close syringe after sample collection. Syringes are taken to the field in an insulated container with an internal temperature of about 20°C. On site, the syringe valve is opened the syringe purged several times to remove stored air and then filled with the desired air sample and sealed by closing the microvalve. In the laboratory, the syringe holding the collected sample is connected to the the inlet of the observation/exposure chamber in which an aliquot of an ozone-sensitive pollen has been incubating in ozone-free air for 2 hr to insure that germination and early tube elongation have occurred.

Pollen may be applied randomly to the agar surface by dusting lightly with a camel's hair brush. This allows for tube growth to take place in a random fashion over the entire agar surface and requires that each tube be measured individually. Pollen may also be applied in straight lines by using the edge of a cover slip as an applicator (*41,42*). This results in straight-line rows of pollen tube growth on either side of the application line. The final configuration appears as one or several lines of differing widths representing the average distance which the pollen tubes have grown out from the line of application. The widths of the lines will differ depending upon the pollen/ozone interaction and then it is only necessary to measure the widths of the line instead of measuring each individual pollen tube.

The chamber may be constructed from a Plexiglas, aluminum, or steel block. It consists of a center well with a bottom made by cementing a circular (or square) #1 cover slip into a recessed ridge. Opposite sides of the chamber block are bored and fitted with two syringe needles with their tips pointing to the center of the chamber. The needles are sealed into the chamber wall so that no gas can leak out between the needle and the chamber walls. The top of the chamber is another cover slip which fits snugly into a second ridge several mm above the lower (bottom) ridge. This produces a chamber with a volume of no more than 2 ml. This volume assures complete flushing of the chamber with 50-100 ml of the air sample to be studied over a 2-hr exposure period. The chamber can be seen in cross section in the schematic drawing of the overall system (Fig. 1) and the actual appearance of the chamber with microscope in place can be seen in the photograph. (Fig. 2).

In practice, a small amount of the agar growth medium is applied in a film to the surface of a #1 cover slip slightly smaller than the well diameter. When the agar has hardened the pollen is applied as previously described and the preparation is incubated for 2 hr at 26°C. The cover slip with germinated pollen is then lowered into the chamber well using a bent forceps. The top cover slip is then cemented temporarily into place using stop cock grease and the syringe containing the air sample to be tested, is connected to the chamber inlet and hooked to a motor driven piston drive which can be programmed to discharge the syringe contents at a desired rate. The piston drive is actuated and the syringe valve opened. The air sample contained in the syringe purges the growth chamber and the pollen tubes are exposed to the air sample for 2 hr. At the end of 2 hr the sample syringe should be empty. The syringe is removed from the chamber inlet, pollen tube growth is recorded by photomicrography and the pollen-covered slip is removed from the chamber, inverted onto a glass slide, sealed with permount, or some other sealant, coded and stored at 4°C for further study, or kept until it is clear that photography was successful.

The photographic negative is then enlarged and the lengths of the tubes measured with a map reader. The tube length frequency distribution is then plotted and the plot compared with a plot of tube length distribution from the same pollen lot exposed to ozone-free charcoal filtered air. If the straight-line method is used, then the width of lines is measured and the width compared.

Results and Discussion

The bar graphs (Figs. 3 and 4) show typical distribution patterns for pollen tube lengths of pollen populations from the ozone-sensitive and ozone-tolerant cultivars of tobacco and petunia. It should be noted that there is a normal distribution of pollen tube lengths (growth) in pollen populations growing without ozone stress. The degree or amount of shift from the normal population (unstressed) distribution which occurs when a sensitive pollen is exposed to an ozone stress is a measure of the sensitivity of that pollen population to ozone, or, conversely, can be used as a measure of the ozone concentration in a particular air sample. By quantifying this shift in distribution of tube lengths from longer to shorter tubes (a shift to the left in the graphs) with known ozone dosages, it should be possible to produce reproducible distribution curves which reflect cultivar pollen behavior under known ozone stresses. When these curves are compared with curves generated by growing pollen in air samples with unknown ozone concen-

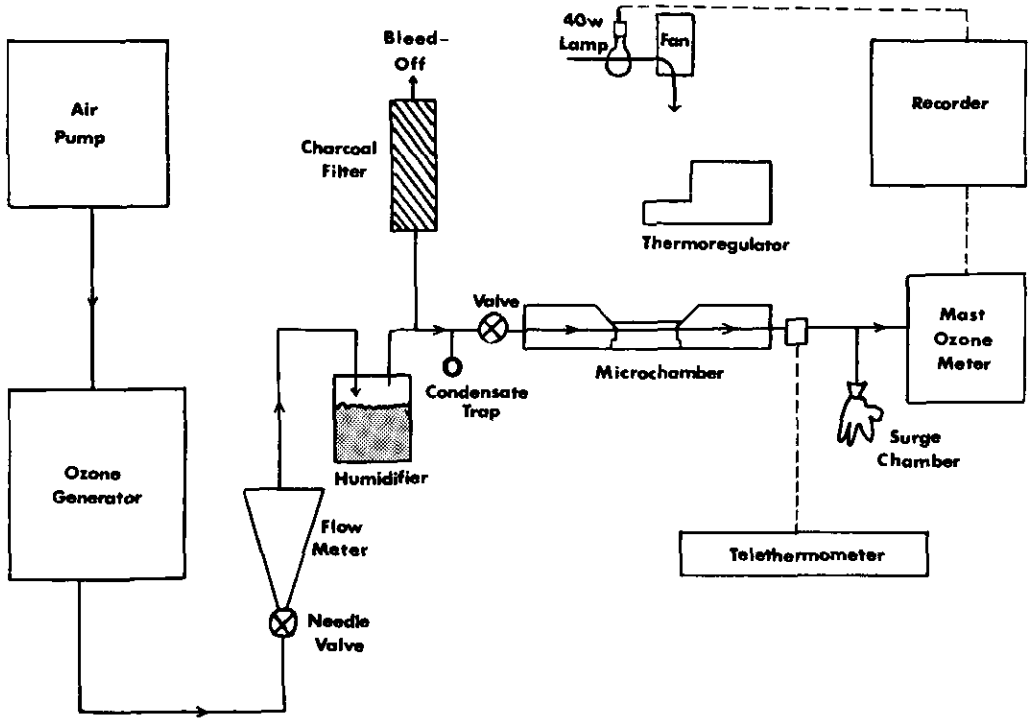

FIGURE 1. Schematic drawing of micro-observation/exposure chamber. Air stream is pulled through pump, passed into ozone generating chamber, charged with ozone, exited over a humidifying surface to the micro-valve through which it passes into the observation/exposure chamber from which it passes into the monitoring probe, is measured by the Mast ozone meter and recorded. Excess ozone/air mixture is exhausted through charcoal-filter chimney.

trations, the differences in distribution patterns can be used as a quantitative assay of the ozone concentrations in the air samples.

To test this, known ozone concentrations were generated in the small macrochamber described earlier in this paper. A range of ozone concentrations was produced and carefully monitored using a calibrated Mast ozone meter. Samples of the ozone/air mixes were withdrawn from the chamber with a 100 ml syringe and injected into the micro chamber as described earlier. Pollen tube growth was photographed, tube lengths measured and the data converted into a series of pollen tube length distribution curves. The curves were superimposed on one another for each ozone concentration and the results are shown in Figures 3 and 4.

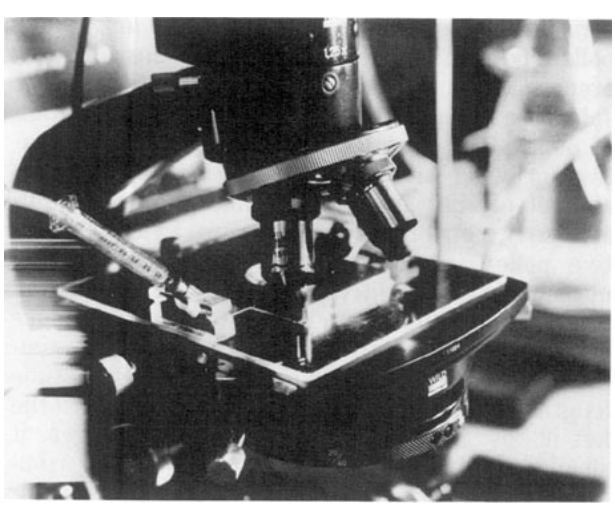

FIGURE 2. Photograph of microscope/block portion of the system showing chamber and inlet port and lower portion of microscope.

The graphs show a good fit of curves when the same cultivar is exposed to the same ozone dose. Differences in ozone concentrations, using the same exposure time, give distinct, reproducible differences in pollen tube length distribution curves. By using pollen from the ozone-sensitive cultivars of tobacco and petunia, cv Bel W-3 and Blue Lagoon, respectively, tube growth distribution curves can be developed for any air sample. The curves generated from these air samples can be compared to standard curves generated for each new pollen batch and the ozone concentration of the air sample can then be established. Use of the above-named cultivars permits to the measurement of an ozone concentration range of 0.04-0.25 ppm by this sys-

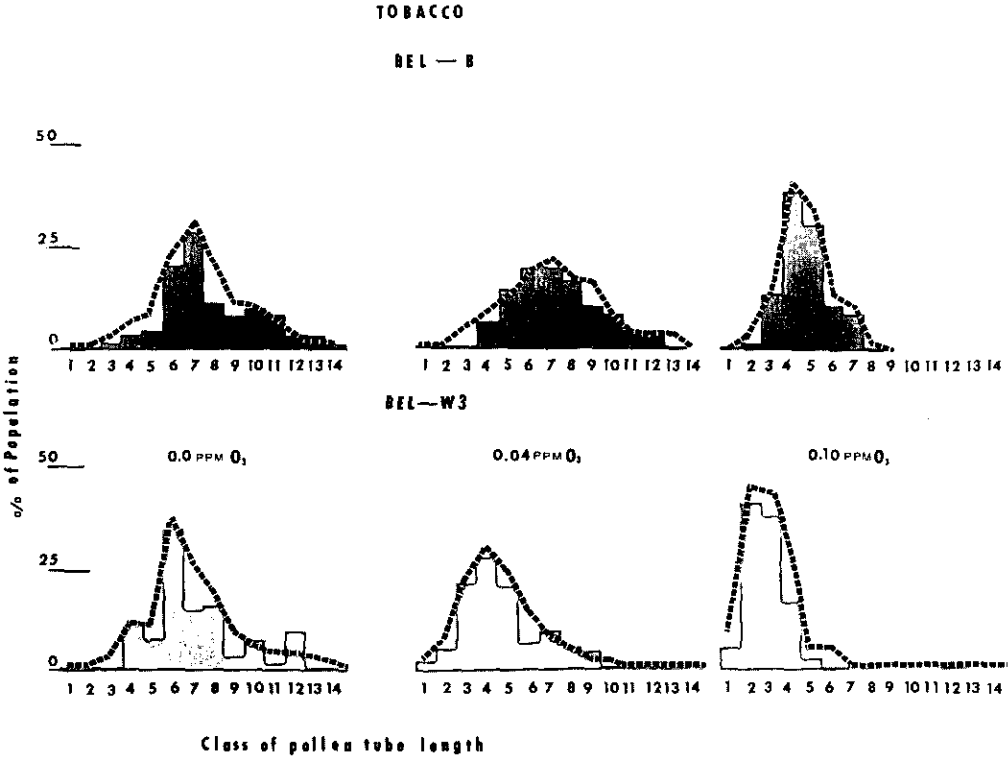

FIGURE 3. Pollen tube length distribution curves generated by exposing growing pollen tubes of ozone-sensitive tobacco, cv Bel W-3 and ozone-resistant tobacco cv Bel B to several ozone dosages. Units are relative. Higher "class" numbers reflect longer tubes. Bars represent mean response and dashed lines show the average deviation from the mean of 10 trials with each ozone concentration/pollen cultivar.

tem. This range is adequate for air quality studies everywhere except in extremely polluted pockets like the Los Angeles, California Air Basin. For such areas, the more ozone-tolerant cultivars of tobacco and petunia, cvs Bel B and Blue Lagoon, respectively, should be used to extend the measureable concentration range to about 0.05 ppm ambient ozone.

The system as described is obviously somewhat simplistic. There are some areas of the world where ozone is the predominant phytotoxic gaseous air pollutant. However, more air masses are likely to contain complex mixtures of gaseous air pollutants, some of which, like SO_2 and HF gas have been demonstrated to exert an effect on the *in vitro* germination and tube growth of pollen species (*1, 4, 6, 7*). There are undoubtedly many organic compounds in the air mix that also affect pollen behavior *in vitro*. The presence in ambient air of these demonstrated and postulated pollen growth inhibitors (and stimulators) suggests that a more realistic use of the pollen bioassay might simply relate *in vitro* pollen growth in "clean" air to *in vitro* pollen growth in ambient air samples, thus describing the quality of the air without attempting to suggest which particular pollutant, or mix of pollutants, was limiting the *in vitro* growth of the pollen. This approach is not very rigorous, but it would suggest the degree of air quality deterioration over a particular site.

A more ambitious approach would involve attempting to discover cultivars whose pollen was affected by a single gaseous pollutant so that an array of pollen sources could be assembled and made available for assessing air quality in terms of such gases as O_3, SO_2, or $CH_2{=}CH_2$, for example. Since great diversity of response to air pollutants is obviously present in plant materials and their pollen populations, the discovery and the selection of specific pollen population monitors is probably feasible and could be carried out in less than 5 years if enough support and interest were generated by the regulatory agencies and the botanical community.

This report is meant to serve as an introduction to the problem of developing a pollen/air pollution bioassay system and as an incentive to expand and refine the system in order to enhance its usefulness to regulatory agencies concerned with the measurement of air quality in a practical, semiquantitative by inexpensive manner.

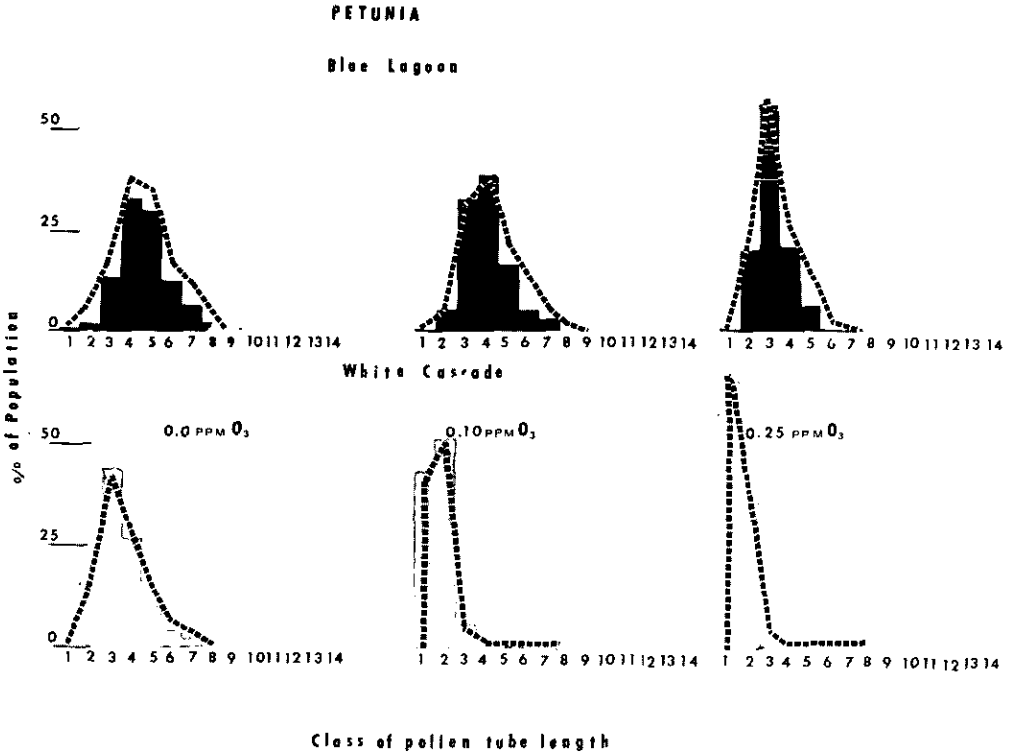

FIGURE 4. Pollen tube length distribution curves generated by exposing growing pollen tubes of ozone-sensitive Petunia cv White Cascade and ozone-resistant Petunia cv Blue Lagoon to several ozone dosages. Units are relative. Higher "class" numbers reflect longer tubes. Bars represent mean response and dashed lines show the average deviation from the mean of 10 trials with each ozone concentration/pollen cultivar.

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